Microdetermination of Unsaturated Disaccharides Produced from Chondroitin Sulfates in Rabbit Plasma by High Performance Liquid Chromatography with Fluorometric Detection

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A highly sensitive high performance liquid chromatographic method with fluorometric postcolumn labeling using 2-cyanoacetamide was developed for determination of unsaturated disaccharides produced enzymatically from chondroitin sulfates (ChS). The calibration curves of the unsaturated disaccharides were linear over the range from 2 pmol to 5 nmol. This method was applied to the analysis of normal rabbit plasma for ChS to verify the sensitivity and practicality.

Keywords Unsaturated disaccharide, chondroitin sulfate, glycosaminoglycan, high performance liquid chromatography, 2-cyanoacetamide

Very small amounts of glycosaminoglycans (GAGs) are reported in mammalian plasma.^{1,2} A major constituent of GAGs is low-sulfated chondroitin 4-sulfate.³ To elucidate the physiological roles of GAGs, many workers have studied analytical methods for determination of GAGs in plasma or serum using chromatography⁴ and electrophoresis.^{3,5,6}

Recently, high-performance liquid chromatographic (HPLC) methods have been developed with UV⁷⁻¹⁰ or fluorometric precolumn labeling method¹¹ after converting GAGs to their corresponding unsaturated disaccharides by enzymatic digestion. Those methods are not suitable for the determination of chondroitin sulfates (ChS) in plasma. UV detection at 232 nm for unsaturated disaccharides is not sensitive enough, and the fluorometric precolumn detection using the 2-aminopyridine derivatives¹¹ requires time-consuming and tedious sample preparation.

In this research, we studied highly sensitive HPLC methods for determination of unsaturated disaccharides, with fluorometric postcolumn labeling using 2-cyanoacetamide, which was introduced by Honda *et al.*¹² Then we tried to apply the method to the analysis of rabbit plasma for small amounts of ChS.

Experimental

Reagents and materials

Standard unsaturated disaccharides (2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose (4Di-OS), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose (4Di-4S) and 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-sulfo-D-galactose (4Di-6S)) and chondroitinase ABC (EC 4.2.2.4) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). 2-Cyanoacetamide was purchased from Kanto Chemicals (Tokyo, Japan), and pronase from Kaken Pharmaceutical Co. (Tokyo, Japan). All other chemicals were of reagent grade. TSK gel NH₂ 60 (Toyo Soda Co., Tokyo, Japan) of particle size 5 μ m was packed in a stainless tube (3 mm i.d. $\times$ 250 mm).

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Apparatus and chromatographic conditions

The chromatographic equipment included a reciprocating pump (PSU-2.5 W, Seishin Pharmaceutical Co., Tokyo, Japan), a double plunger pump for the reagent solution (SPU-2.5 W, Shimamura instrument Co., Tokyo, Japan), a sample injector (VMD-350, Shimamura instrument Co.), a fluoromonitor (RF-530 fluorescence spectromonitor, Shimadzu Seisakusho, Kyoto, Japan), a variable input recorder (SS-250F, Sekonic Co., Tokyo, Japan) and a dry reaction bath (Type DB-3, Shimamura Instrument Co.). A UV monitor (UVILOG-7, Oyo-Bunko Kiki Co., Tokyo, Japan) was used for the detection at 232 nm.

Established HPLC conditions were as follows: A 10 μ l portion of sample solution was loaded *via* a sample injector with a 20 μ l loop. A TSK gel NH₂ 60 column (3.0 mm i.d. $\times$ 250 mm) was eluted with 10 mM ammonium formate buffer (pH 5.0) containing 10 mM sodium sulfate in 4% acetonitrile at a flow rate of 0.5 ml/min. To the eluate were added 0.125 M sodium tetraborate-sodium hydroxide buffer (pH 10.8) and aqueous 1% 2-cyanoacetamide solution at a flow rate of 0.2 ml/min by using a double-plunger pump. The mixture passed through a polytetrafluoroethylene

(PTFE) reaction coil (0.5 mm i.d.×10 m) set in a dry reaction bath thermostated at 100°C and a following PTFE cooling coil (0.25 mm i.d.×2 m) in a water bath at 25°C. The effluent was monitored by the fluoromonitor (Ex. 346 nm, Em. 410 nm).

Collection of rabbit plasma

Blood was collected from the marginal ear vein of adult rabbits. Ethylenediaminetetraacetic acid disodium salt was added to the blood as an anticoagulant. The blood was separated into plasma and cells by centrifugation at 1200×g for 15 min; the plasma was then transferred to a tube.

Preparation of plasma GAGs

Plasma GAGs were separated by a modification of the method of Emura and Mukuda¹³ as follows: To 100 µl of rabbit plasma, 40 µl of 0.05 M Tris-HCl buffer (pH 8.0) containing 1% pronase was added, and the mixture was incubated at 45°C for 3 h. To the solution, 340 µl of 12% sodium chloride solution and 60 µl of 0.1 M acetic acid solution were added. Then the mixture was heated in a boiling water bath for 5 min. After being cooled in a water bath, the solution was centrifuged at 2300×g for 15 min. To 360 µl of the supernatant, 40 µl of 0.1 M sodium hydroxide solution and 1.6 ml of cooled ethanol saturated with sodium acetate were added. The mixture was left to stand at 5°C for 1 h and centrifuged at 2300×g for 15 min. To the precipitate was added 0.6 ml of 0.015% cetylpyridinium chloride containing 0.03 M sodium chloride warmed at 37°C before use. The mixture was left to stand overnight at 0°C. The solution was centrifuged at 2300×g for 15 min at 5°C. The precipitate was washed twice with 0.6 ml of ethanol saturated with sodium chloride, and then lyophilized.

Enzymatic digestion

Digestion of plasma GAGs with chondroitinase ABC was carried out according to the method¹⁴ of Kodama *et al.*

Results and Discussion

Chromatographic separation and detection of unsaturated disaccharides

Separation of Δ Di-0S, Δ Di-4S and Δ Di-6S was successfully performed by some workers on amino-silica type columns with acidic (pH 4–6)^{8,10} eluents. Considering the postcolumn method with 2-cyanoacetamide which is reacted with the unsaturated disaccharides in weakly alkali solution, the concentration of salts in an eluent was decreased to change the pH of effluent. The unsaturated disaccharides were resolved within 20 min on the TSK gel NH₂ 60 column with 10 mM ammonium formate buffer (pH 5.0) containing 10 mM sodium sulfate in 4% acetonitrile as an eluent (Fig. 1). The detection system was set by the modi-

fication of Honda's method¹² using 2-cyanoacetamide as a detection reagent. The fluorescence intensities of unsaturated disaccharides were examined by a flow injection system without the separation column. The excitation and emission wavelengths were set to 346 nm and 410 nm, respectively, for the determination of unsaturated disaccharides, while neutral sugars had the maximum fluorescence intensities at 331 nm (Ex.) and 383 nm (Em.). As shown in Table 1, relative fluorescence intensities of Δ Di-0S, Δ Di-4S and Δ Di-6S

Table 1 Comparison of fluorescence intensities formed from various carbohydrates using flow injection analysis

Carbohydrate	Relative molar fluorescence intensity
	(Ex. 346 nm)/(Em. 410 nm)
D-Glucose	1
D-Galactose	1
D-Glucosamine HCl	7
D-Galactosamine HCl	4
D-Glucuronic acid	7
D-Galacturonic acid	19
N-Acetyl-D-glucosamine	22
N-Acetyl-D-galactosamine	61
Δ Di-0S	398
Δ Di-4S	436
Δ Di-6S	456

Fig. 1 Typical chromatograms of standard unsaturated disaccharides. A, UV detection at 232 nm (The separation system is the same as that in the postcolumn labeling method (B).); B, fluorometric detection using 2-cyanoacetamide (The other conditions are shown in the experimental section.); sample size, 10 µl (50 ng of each sugar). Peaks: 1, Δ Di-0S; 2, Δ Di-6S; 3, Δ Di-4S.

were about 300–400 times stronger than those of neutral sugars in our system.

Calibration curves for Δ Di-0S, Δ Di-4S and Δ Di-6S were linear in the ranges of 2 pmol–5 nmol, 4 pmol–

5 nmol and 4 pmol–5 nmol respectively. The sensitivity of this method was about 20 times higher than that of UV detection as shown in Fig. 1. The relative standard deviations ($n=7$) of Δ Di-0S, Δ Di-4S and Δ Di-6S were 1.5, 1.4 and 1.8%, respectively.

Fig. 2 Chromatograms of unsaturated disaccharides produced from rabbit plasma GAGs. A, UV detection at 232 nm; B, fluorometric detection using 2-cyanoacetamide; sample size, 10 μ l. Peaks: 1, Δ Di-0S; 2, Δ Di-6S; 3, Δ Di-4S.

Analysis of rabbit plasma ChS

The present method was applied to the determination of unsaturated disaccharides produced from normal rabbit plasma ChS by chondroitinase ABC digestion. Figure 2 shows typical chromatograms of unsaturated disaccharides produced from plasma ChS. The peaks of Δ Di-0S, Δ Di-4S and Δ Di-6S were detected with postcolumn labeling using 2-cyanoacetamide (Fig. 2B), while they could not be detected with UV detection at 232 nm (Fig. 2A). The unsaturated disaccharides mainly produced from plasma ChS were Δ Di-0S and Δ Di-4S. This result indicates that low-sulfated chondroitin 4-sulfate is present in plasma.

Variation of ChS concentration in a rabbit plasma within a month were estimated from the amounts of Δ Di-0S, Δ Di-4S and Δ Di-6S (Table 2). The data shows that concentrations of plasma ChS are fairly constant in a rabbit. Moreover, analytical results of ChS concentrations in rabbit plasma are shown in Table 3. It is very interesting that no differences of ChS concentrations could be found among individual rabbits. The occurrence of low-sulfated chondroitin 4-sulfate, which is composed of both Δ Di-0S and Δ Di-4S, is of interest in particular with regard to the process

Table 2 Variation of unsaturated disaccharides produced from chondroitin sulfates in a normal rabbit plasma after digestion with chondroitinase ABC

Plasma	Unsaturated disaccharide/ μ M			Total amount/ μ M
	Δ Di-0S	Δ Di-4S	Δ Di-6S	
A-1	9.30 (51.7)	8.25 (45.8)	0.45 (2.5)	18.00
A-2	9.40 (49.9)	9.00 (47.7)	0.45 (2.4)	18.85
A-3	9.00 (47.2)	9.60 (50.3)	0.47 (2.5)	19.07
A-4	8.70 (47.6)	9.10 (49.8)	0.47 (2.6)	18.27
A-5	8.10 (46.3)	9.00 (51.4)	0.41 (2.3)	17.51
Mean $\pm$ S.D.	8.90 $\pm$ 0.47	8.99 $\pm$ 0.43	0.45 $\pm$ 0.02	18.34 $\pm$ 0.56

The values in the parentheses represent the proportions (%) of the disaccharide to the total amount of unsaturated disaccharides.

Table 3 Determination of unsaturated disaccharides produced from chondroitin sulfates in normal rabbit plasma after digestion with chondroitinase ABC

Rabbit	Unsaturated disaccharide/ μ M			Total amount/ μ M
	Δ Di-0S	Δ Di-4S	Δ Di-6S	
A	9.30 (51.7)	8.25 (45.8)	0.45 (2.5)	18.00
B	10.76 (57.9)	7.45 (40.1)	0.38 (2.0)	18.59
C	11.12 (55.2)	8.2 (41.1)	0.76 (3.8)	20.14
D	9.15 (48.7)	8.89 (47.3)	0.74 (4.0)	18.78
E	9.72 (45.1)	11.11 (51.5)	0.74 (3.4)	21.57
Mean $\pm$ S.D.	10.01 $\pm$ 0.79	8.79 $\pm$ 1.25	0.61 $\pm$ 0.16	19.42 $\pm$ 1.28

The values in the parentheses represent the proportions (%) of the disaccharide to the total amount of unsaturated disaccharides.

of sulfation.

As described above, this sensitive chromatographic method was first applied to the determination of ChS in small amounts of plasma sample. This method is effective for the determination of trace amounts of hyaluronic acid, heparan sulfate and keratan sulfate after the specific enzymatic digestion.

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